Dronedarone reduces arterial thrombus formation

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ORIGINAL CONTRIBUTION

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Alexander Breitenstein · Susanna H. M. Sluka · Alexander Akhmedov · Simona Stivala · Jan Steffel · Giovanni G. Camici · Huy H. Riem · Hans-Jurg Beer · Jan-Dirk Studt · Firat Duru · Thomas F. Luscher · Felix C. Tanner

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Keywords Dronedarone · Arterial thrombosis · Animal model · Platelets

A. Breitenstein and S. H. M. Sluka contributed equally to this work.

A. Breitenstein · J. Steffel · G. G. Camici · F. Duru · T. F. Luscher · F. C. Tanner (✉)
Cardiology, Cardiovascular Center, University Hospital Zurich, Raemistrasse 100, 8091 Zurich, Switzerland
e-mail: felix.tanner@access.uzh.ch

A. Breitenstein · S. H. M. Sluka · A. Akhmedov · S. Stivala · J. Steffel · G. G. Camici · F. Duru · T. F. Luscher · F. C. Tanner
Cardiovascular Research, Physiology Institute, University of Zurich, Zurich, Switzerland

A. Breitenstein · S. H. M. Sluka · A. Akhmedov · S. Stivala · J. Steffel · G. G. Camici · F. Duru · T. F. Luscher · F. C. Tanner
Center for Integrative Human Physiology (ZIHP), University of Zurich, Zurich, Switzerland

H. H. Riem
Cardiovascular Therapy Research Laboratory, Center for Clinical Research, University Hospital Zurich, Zurich, Switzerland

H.-J. Beer
Cantonal Hospital Baden, Baden, Switzerland

J.-D. Studt
Hematology, University Hospital Zurich, Zurich, Switzerland

Introduction

Atrial fibrillation (AF) is the most common sustained arrhythmia in the Western world and represents a major health burden [10, 12]. Management of patients with AF focuses either on rhythm-control or rate-control strategies as well as the on prevention of thromboembolic events. The most commonly used anti-arrhythmic drug is amiodarone; nevertheless, its use is restricted due to its wide range of adverse side effects such as thyroid and pulmonary toxicity. Dronedarone, a benzofuran derivative with electrophysiological properties similar to amiodarone, was therefore designed to function as an alternative to amiodarone [25]. In several clinical trials, dronedarone delayed the time to first recurrence of AF and also decreased the

number of recurrences [14, 19, 24]. Indeed, in the dronedarone trial to asses the prevention of cardiovascular hospitalization or death in patients with AF (ATHENA), dronedarone treatment was associated with a significant reduction in first cardiovascular hospitalisations as well as death from cardiovascular causes; interestingly, there were also fewer first hospitalisations for acute coronary syndromes (ACS; $P = 0.03$ for the dronedarone-treated group versus control) [14]. Whether this is only due to the anti-ischemic effect of lower ventricular heart rate or a result of an additional mechanism such as direct inhibition of thrombus formation currently remains unknown.

This study was therefore designed to investigate whether dronedarone, in addition to its electrophysiological properties, inhibits arterial thrombus formation.

Materials and methods

Dronedarone plasma and tissue concentrations

Concentrations of dronedarone in mouse plasma and thoracic aorta were measured by high performance liquid chromatography [HPLC; LiChrospher®60, 5C18; 250 × i.d. 4 mm; mobile phase: methanol:water:25 % NH_3 (89.47:8.77:1.76 w/w); flow rate: 1 mL/min]. Aorta (25 mg) was homogenized in 0.5 mL methanol at 4 °C using a Potter S glass homogenizer (Braun, Melsungen, Germany). After centrifugation at 3,000*g*, the pellet was washed twice with 0.25 mL methanol. The organic extracts were pooled and evaporated to dryness (Speed Vac, Savant Instruments Inc., Farmingdale, NY, USA). The residue was dissolved in 75 µL water, and an aliquot of 50 µL was analyzed. Plasma (200 µL) was mixed with 0.1 mL 0.5 mM KH_2PO_4 and extracted three times with 0.75 mL diethylether. The organic extracts were pooled and evaporated to dryness as described. The residue was dissolved in 100 µL of methanol, and an aliquot of 50 µL was used for HPLC.

Carotid artery thrombosis model

Twelve-week-old C57Bl/6 mice were divided into two groups: dronedarone (200 mg/kg body weight with a once daily oral gavage for 14 days) or control (1.4 % methylcellulose). Twenty-four hours after the last application, mice were anesthetized by intraperitoneal injection of 87 mg/kg sodium pentobarbital (Butler, Columbus, OH, USA). Rose bengal (Fisher Scientific, Fair Lawn, NJ, USA) was diluted to 12 mg/mL in phosphate-buffered saline and then injected into the tail vein at a concentration of 63 mg/kg. Mice were secured in a supine position, placed under a dissecting microscope, and the right common carotid artery was exposed following a midline cervical incision. A Doppler flow probe (Model 0.5 VB, Transonic Systems, Ithaca, NY, USA) was applied and connected to a flowmeter (Transonic, Model T106). 6 min after rose bengal injection, a 1.5-mW green light laser (540 nm; Melles Griot, Carlsbad, CA, USA) was applied to the site of injury at a distance of 6 cm for 60 min or until thrombosis occurred. From the onset of injury, blood flow was monitored up to 120 min, at which time the experiment was terminated [4]. Occlusion was defined as flow ≤0.1 mL/min for at least 1 min.

Platelet count

Platelets were counted by flow cytometry using whole blood collected in EDTA tubes (B&D Diagnostics, Franklin Lakes, NJ, USA).

Reticulated platelet staining

Reticulated platelets are stained by mixing 5 µL of EDTA-anticoagulated blood with 50 µL Thiazole Orange solution (0.1 µg/mL in PBS). Samples are incubated 15 min at room temperature and then fixed by adding 1 % PFA in PBS, and immediately analyzed on a FacsCanto (BD Bioscience). Platelets are gated based on their FSC/SSC characteristics and % positive platelets determined with BD FacsDiva software.

Glycocalicin index

Plasma glycocalicin is determined by enzyme linked immunosorbent assay (ELISA). Briefly, flat-bottom 96-well plates (Costar) are coated overnight with an antibody against the extracellular domain of murine GPIbα (generous gift of B. Nieswandt), overnight at 4 °C. Wells are then washed with PBS-Tween 0.1 % and blocked with 5 % milk-5 % BSA in PBS for 2 h at 37 °C. After washing, they are incubated with duplicate standard (pooled mouse plasma) and samples at 1:10 and 1:20 dilution in 1 % milk–1 % BSA at 37 °C for 1 h. Wells are washed and incubated with a second antibody against GPIbα conjugated to HRP for 30 min. After washing, wells are incubated with TMB substrate reagent (BD Bioscience) for 5 min, and reaction is stopped by adding 0.5 M sulphuric acid. Optical densities are read at 450 nm on a Versamax plate reader (Molecular Devices) and glycocalicin concentrations are calculated by the SoftMax Pro software based on the standard curve. Glycocalicin index is calculated by multiplying the plasma glycocalicin concentration for the platelet count normalized to 250,000/µL.

Platelet aggregation studies

Platelet aggregation was studied using a Chrono-Log whole blood impedance aggregometer (Chrono-Log, Havertown, PA, USA). For studies performed in murine platelets, citrated blood was drawn by puncture from the right ventricle. Aggregation studies were performed with citrated blood within 1 h. Platelets were equilibrated under constant stirring for 1 min prior to addition of human thrombin (0.5 U/mL; Sigma Aldrich, Frankfurt, Germany), ADP (20 mU/mL; Chrono-Log), equine collagen type 1 (5 μg/mL; Chrono-Log), ADP (20 μM; Chrono-Log), or botrocetin (0.5 mg/mL; Sigma Aldrich). Aggregation was displayed as a function of time (AGGRO/LINK® Software; Chrono-Log). Results were expressed as maximal aggregation (Ω), and lag time (s).

Coagulation times

Prothrombin time (PT) and activated partial thromboplastin time (aPTT) were assessed by the Start4 analyzer (Diagnostica Stago, Paris, France) with according reagents (Roche Diagnostics, Basel, Switzerland). Plasma from citrated blood (3.8 % citrate 1/10) was extracted by 10 min centrifugation (2,100*g*, 4 °C), and stored immediately at −80 °C until analyzed.

Vessel wall mRNA and protein analysis

Total RNA was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA was generated using Ready-To-Go You-Prime First-Strand Beads (Amersham Bioscience, Glattbrugg, Switzerland) and first-strand cDNA primer. Quantitative real-time polymerase chain reaction was performed using SybrGreen Jump start kit (Sigma Aldrich). Primers were designed to detect mouse TF, TFPI and PAI1. Data were normalized for murine L28 and analyzed by the comparative ΔC_T method.

For protein analysis, aortas of mice were homogenized in 50 mL of lysis buffer (50 mmol Tris–HCl, 100 mmol NaCl, 0.1 % Triton X-100, pH 7.4) and left to stand on ice for 30 min. One hundred microgram samples were loaded and separated by 10 % SDS-PAGE; proteins were transferred to a PVDF membrane and probed with an antibody to PAI1 (1:1,000 dilution). Equal protein loading was determined by staining for α-tubulin (1:10,000 dilution).

Statistical analysis

Data are indicated as mean ± SEM. For statistical analysis, one-way ANOVA was performed with a post hoc analysis. A P value <0.05 denoted a significant difference.

Table 1 Liver and kidney function were not affected by dronedarone treatment

	Control ($n = 7$)	Dronedarone ($n = 7$)
AST (U/L)	89.1 ± 15.2	78.9 ± 6.9
ALT (U/L)	23.2 ± 1.8	28.9 ± 1.3
Creatinine (μmol/L)	18.9 ± 1.3	19.5 ± 1.1

AST alanine transaminase, *ALT* aspartate transaminase

Results

Dronedarone inhibits arterial thrombus formation at a clinically relevant plasma concentration

Mice were treated with either dronedarone (200 mg/kg body weight once daily) or vehicle, respectively. Dronedarone plasma concentration was 0.12 ± 0.014 μg/mL, while the tissue level reached a concentration of 10.28 ± 2.18 ng/mg ($n = 3$; data not shown). Alteration in liver or kidney function during the treatment period was not observed since liver enzymes [alanine transaminase (ALT), aspartate transaminase (AST)] as well as serum creatinine were similar in treated and control mice ($n = 7$; P = NS; Table 1).

Mean occlusion time due to thrombus formation equaled 27.9 ± 4.2 min in control mice as compared to 50.9 ± 8.9 min in dronedarone-treated mice ($n = 7$; $P < 0.05$; Fig. 1a). Real-time blood flow measurement demonstrated that the two curves separated after 15 min (Fig. 1b). In contrast, initial blood flow in the carotid artery and heart rate did not differ between vehicle- and dronedarone-treated animals ($n = 7$; P = NS; Table 2).

Dronedarone reduces platelet aggregation, but not platelet turnover

Maximal platelet aggregation induced by thrombin or collagen was impaired in dronedarone-treated animals as compared to controls ($n = 11$; $P < 0.05$; Fig. 2a, c). Time to begin of aggregation (lag time) in response to thrombin or collagen tended to be prolonged after dronedarone treatment ($n = 11$; $P = 0.08$; Fig. 2b, d). In contrast, ADP and botrocetin-induced platelet aggregation was not altered by dronedarone as compared to controls ($n = 4$–11; P = NS; Table 3).

Total platelet number ($n = 7$; P = NS; Fig. 3a) as well as platelet turnover remained unaffected by dronedarone. Turnover was analyzed by measuring the RNA content of platelets as a marker for immature platelets, and platelet degradation was assessed by determining the glycocalicin index. Neither the level of immature platelets ($n = 5$; P = NS; Fig. 3b) nor platelet degradation ($n = 6$;

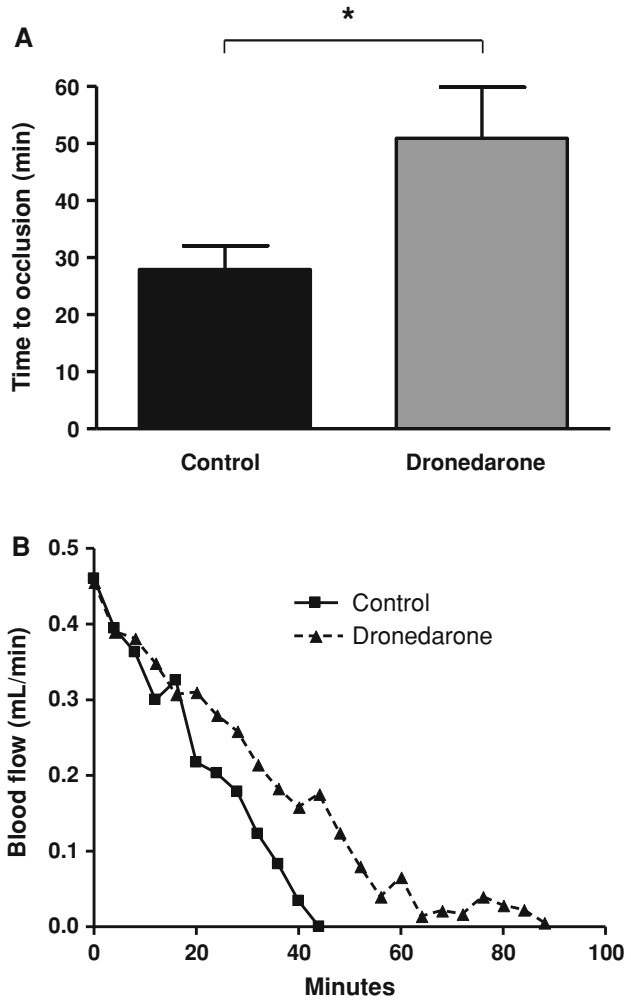

Fig. 1 Dronedarone inhibits arterial thrombus formation. **a** Time to thrombotic occlusion after mouse carotid artery photochemical injury in vivo. Dronedarone delayed thrombus formation (*$P < 0.05$ vs. control). **b** Blood flow recordings of control and dronedarone-treated mouse during photochemical injury in vivo. Laser injury was initiated at time point 0 min

P = NS; Fig. 3c) was altered in the presence of dronedarone.

Expression of plasminogen activator inhibitor-1 (PAI1), but not tissue factor (TF) or TF pathway inhibitor (TFPI), is reduced by dronedarone treatment

Dronedarone significantly reduced PAI1 expression, an important anti-fibrinolytic factor, both at the mRNA and the protein level ($n = 6$–8; $P < 0.05$; Fig. 4a, b). In contrast, expression of tPA, a pro-fibrinolytic parameter, remained unchanged ($n = 6$–7; P = NS; Fig. 4c). Similarly, the level of TF mRNA, the key trigger of the coagulation cascade, as well as its physiological inhibitor, TF pathway inhibitor (TFPI), were not altered by dronedarone treatment ($n = 7$; P = NS; Table 4).

Table 2 Hemodynamic parameters were not altered by dronedarone

	Control ($n = 7$–10)	Dronedarone ($n = 7$–10)
Initial blood flow (mL/min)	0.47 ± 0.048	0.47 ± 0.025
Heart rate (bpm)	320 ± 48	310 ± 81

Dronedarone does not alter coagulation times

Prothrombin time (PT) and activated partial thromboplastin time (aPTT) remained unaffected by dronedarone treatment ($n = 7$–10; P = NS; Table 4).

Discussion

This study demonstrates that dronedarone impairs thrombus formation at plasma concentrations similar to those observed in humans. These data reveal a novel pleiotropic action of dronedarone, which offers an explanation for the previously observed beneficial clinical effects of this substance. The effect on thrombosis was paralleled by an inhibition of platelet aggregation as well as PAI1 expression, whereas no direct effect of dronedarone on the expression of coagulation factors including TF was observed. Hence, the antithrombotic effect of dronedarone described herein is most probably due to an inhibition of both platelet aggregation and activation of fibrinolysis.

Patients receiving the usual dronedarone treatment (400 mg orally twice a day) exhibit peak steady state plasma concentrations between 85 and 150 ng/mL [7]. Hence, even though the dronedarone doses applied in our study are higher as compared to humans, the dronedarone plasma concentrations measured are within the range reached in patients. Thus, the observed anti-thrombotic effect of dronedarone occurs under clinically relevant conditions. Furthermore, in this animal study, there were no signs of early dronedarone toxicity since liver enzymes and creatinine level were not changed.

ACS is caused by thrombus formation, which results from an imbalance between procoagulant factors, platelet activation and fibrinolysis [18]. Most commonly, a coronary is formed on top of a ruptured atherosclerotic plaque [23], which leads to the exposure of highly procoagulant plaque material activating the coagulation system and platelets [2, 22]. Both TF and platelets are thereby crucially involved since TF is the key activator of the coagulation cascade [1], and inhibition of platelets represents the main goal in acute coronary syndromes. On the other hand, PAI1 and tPA regulate the fibrinolytic system and may promote thrombus degradation [9, 15]. The anti-thrombotic effect of dronedarone was paralleled by a reduced platelet activation

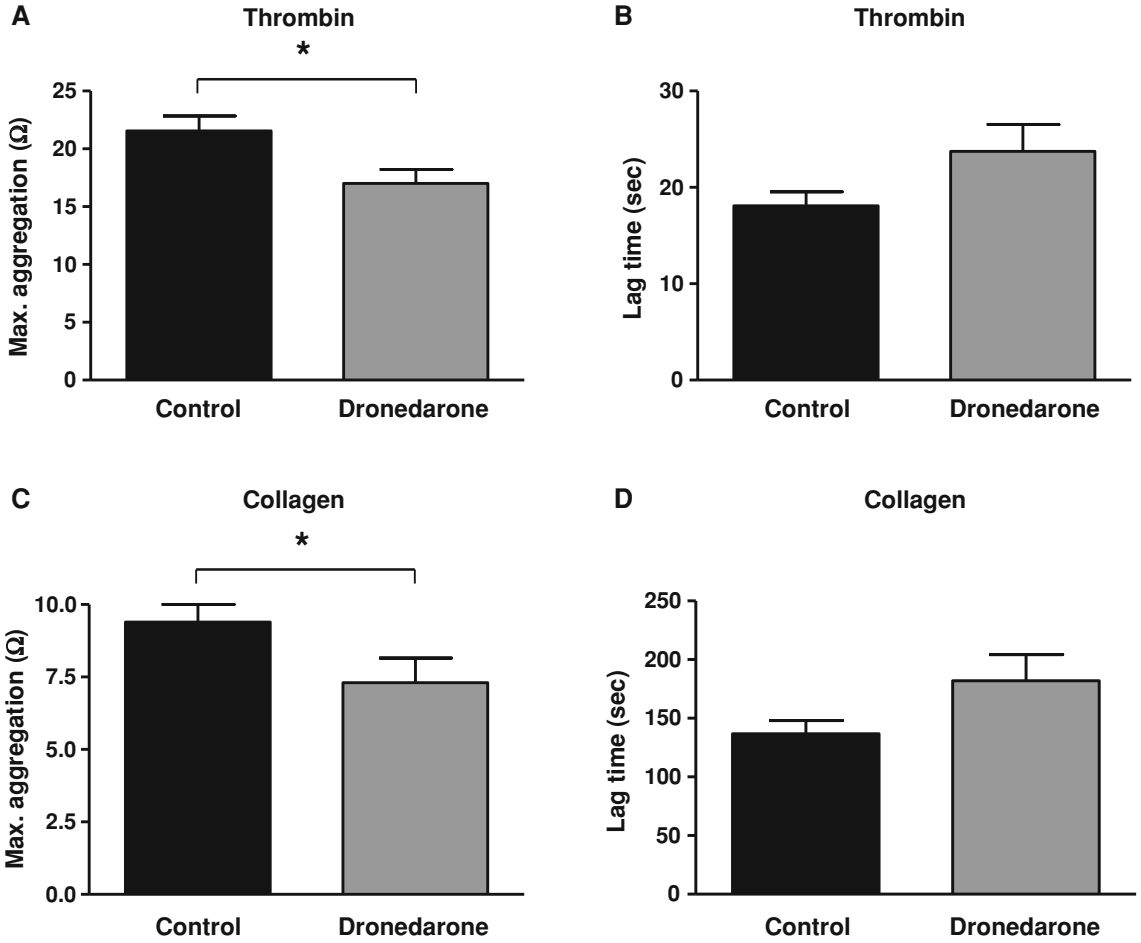

Fig. 2 Dronedarone inhibits platelet aggregation. **a–d** Dronedarone reduced platelet aggregation in thrombin- and collagen-induced activation (*$P < 0.05$), whereas lag time did not differ between the two groups ($P = 0.08$)

Table 3 ADP and botrocetin-induced platelet aggregation was not altered by dronedarone

	Control ($n = 4$–9)	Dronedarone ($n = 7$–10)
ADP, max. aggregation (Ω)	3.4 ± 0.9	3.3 ± 0.96
ADP, lag time (s)	249 ± 65	321 ± 101
Botrocetin, max. aggregation (Ω)	6.8 ± 0.8	9.8 ± 1.1
Botrocetin, lag time (s)	141 ± 25	116 ± 16

as well as by a diminished PAI1 expression; hence, dronedarone impairs thrombus formation by a dual action on two critical events involved in arterial thrombus formation. Indeed, the importance of platelets in arterial thrombus formation is well known, and inhibition of platelet aggregation represents the main target in the medical treatment of ACS. Moreover, a role for PAI1 in the regulation of arterial thrombus formation has been described in vivo [9, 15]; and higher PAI1 levels have been detected in the atherosclerotic plaque from patients with coronary artery disease [21]. Nevertheless, the present data reveal an association between PAI1 and thrombus formation, and the relative contribution of PAI1 reduction versus platelet inhibition to the antithrombotic effect remains speculative. Even though a vasodilatory effect of dronedarone has been described in pigs [11], an alteration in hemodynamic parameters having the potential to influence the results of the thrombosis model can be excluded because initial blood flow and heart rate did not differ between the control and dronedarone groups.

Dronedarone impaired platelet aggregation due to stimulation with thrombin and collagen, whereas it did not aggregate in response to ADP and botrocetin. The cellular effects of thrombin and ADP receptors—similar to other G-protein coupled receptors—are mediated by cAMP. Dronedarone's pattern of action therefore suggests that it interferes with platelet aggregation at the receptor level. Such an effect has been observed for other mediators as well. The most relevant example is given by clopidogrel, a potent ADP-receptor inhibitor, selectively interfering with ADP-induced platelet aggregation [26]. Similarly, bivalirudin inhibits platelet aggregation in response to thrombin

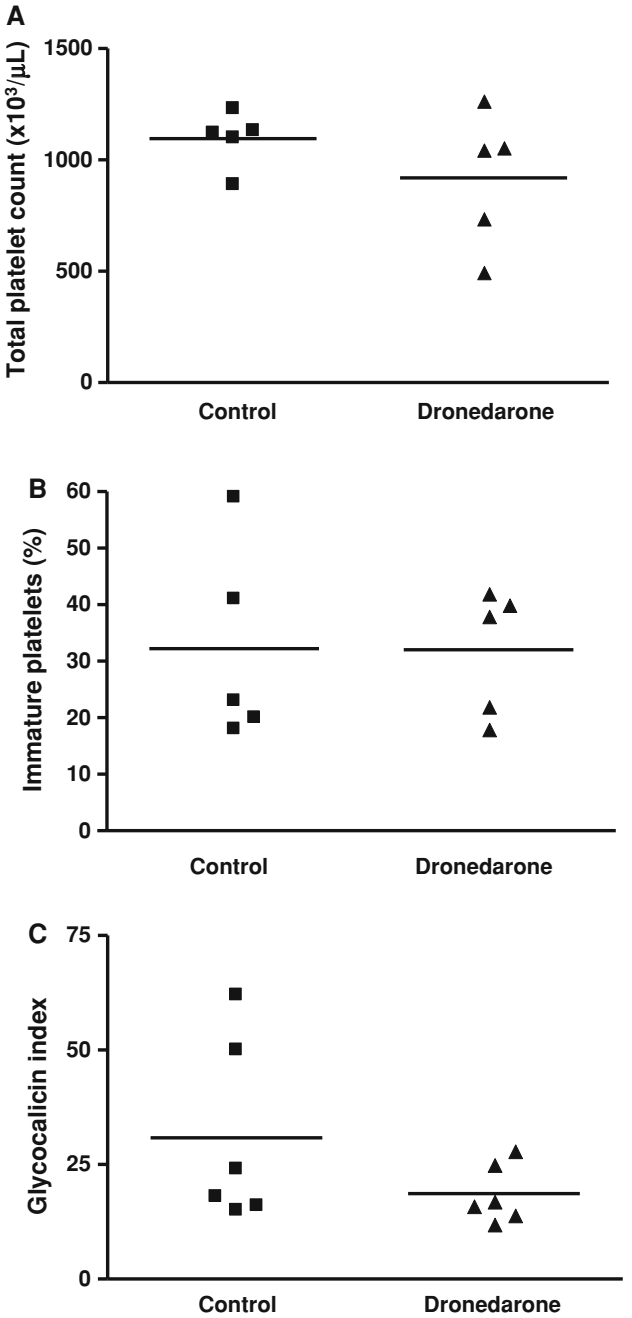

Fig. 3 Dronedarone does not change platelet turnover. **a** Total platelet number did not differ between the two groups (P = NS). **b** Dronedarone did not change the number of immature platelets (P = NS). **c** Glycocalicin index was not altered after dronedarone treatment (P = NS)

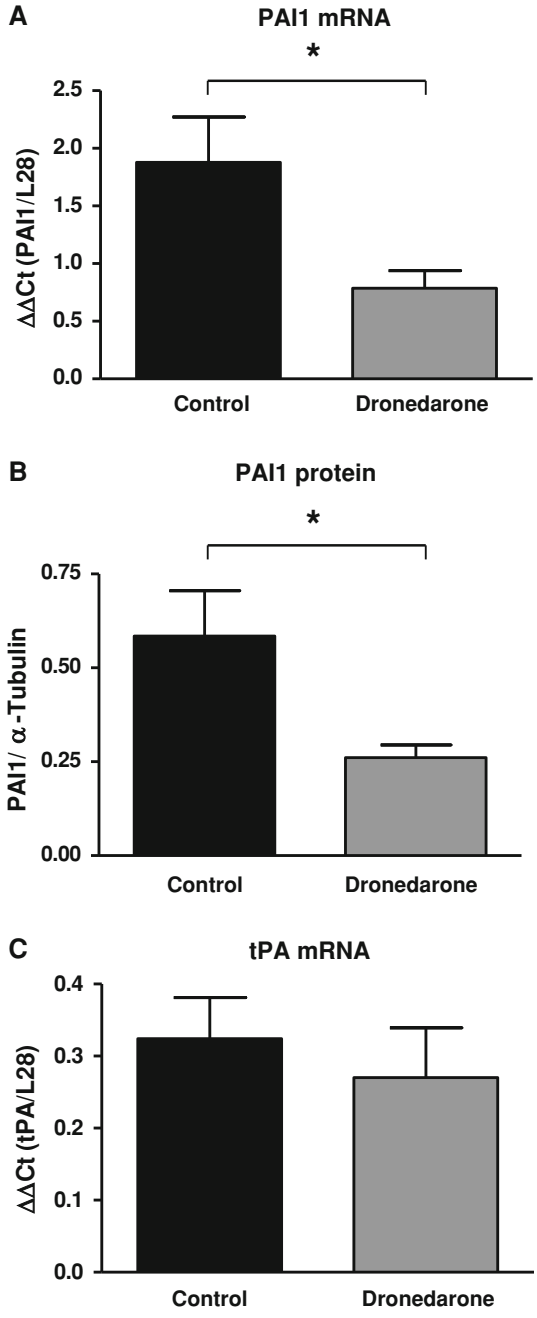

Fig. 4 Dronedarone inhibits PAI1 expression in the vessel wall. **a**, **b** PAI1 mRNA and protein expression in the arterial wall. Dronedarone reduced PAI1 expression (*$P < 0.05$). **c** tPA expression in the vessel wall was not changed by dronedarone (P = NS)

and collagen without affecting other agonists [16]. Hence, like other drugs, dronedarone inhibits platelet aggregation by interfering with activation of specific receptors.

An overview about the potential antiarrhythmic effects of dronedarone was recently published [6]. Dronedarone has been shown to reduce time to first recurrence in patients with paroxysmal atrial fibrillation as well as the rate of first admission for cardiovascular events or death from any cause [14]. Trials investigating the role of dronedarone in heart failure patients failed to confirm these results in this patient group [17]. In additional to these antiarrhythmic effects, dronedarone exerts various pleiotropic actions. Mice pretreated with dronedarone exhibited a reduced myocardial infarction in a ligation ischemia model, probably due to attenuation of calcium overload

Table 4 Coagulation times and parameters were equal between the control and the dronedarone group

	Control ($n = 7$–10)	Dronedarone ($n = 7$–10)
TF expression ($\Delta\Delta C_T$ TF/L28)	0.087 ± 0.022	0.080 ± 0.017
TFPI expression ($\Delta\Delta C_T$ TFPI/L28)	0.11 ± 0.029	0.09 ± 0.026
PT (s)	11.99 ± 1.23	12.70 ± 1.05
aPTT (s)	28.11 ± 2.45	20.20 ± 3.52

TF tissue factor, *TFPI* tissue factor pathway inhibitor, *PT* prothrombin time, *aPTT* activated partial thrombin time

during ischemia [13, 20]. Similarly, dronedarone pretreatment reduced the area of cerebral infarction in rats [8, 13], and the expression of ischemia-related genes after rapid atrial pacing was impaired in pigs treated with the drug resulting in prevention of ventricular microcirculatory abnormalities [3]. Hence, besides the pleiotropic anti-thrombotic effect described in this study, other beneficial effects of this kind have been observed for dronedarone.

In the ATHENA trial (A Placebo-Controlled, Double-Blind, Parallel Arm Trial to Assess the Efficacy of Dronedarone 400 mg bid for the Prevention of Cardiovascular Hospitalisation or Death from Any Cause in patients with Atrial Fibrillation), 4,628 patients were randomized to either dronedarone (400 mg bid) or placebo [14]. In these patients suffering from atrial fibrillation, dronedarone reduced the combined endpoint of hospitalisation for cardiovascular causes and cardiovascular death, which was driven primarily by the reduction in cardiovascular hospitalisations. Furthermore, first hospitalisations for acute coronary syndromes (62 in the dronedarone group vs. 89 in the placebo group, $P = 0.02$) were significantly lower in the dronedarone group. While the reduced number of first hospitalisations due to atrial fibrillation is likely caused by the anti-arrhythmic effect of dronedarone, the reason for the decrease in hospitalisations for acute coronary syndromes is not that obvious. A lower heart rate and thereby anti-ischemic effect may play a role, but an additional pleiotropic anti-thrombotic effect, as described in the present study, cannot be ruled out. In contrast, the PALLAS trial (Permanent Atrial Fibrillation Outcome Study Using Dronedarone on Top of Standard Therapy) [5] demonstrated a higher rate of adverse cardiovascular events. The reason for the opposite outcome between ATHENA and PALLAS has widely been discussed, but remains unknown, in particular since direct comparison of the two studies is difficult. Patients in PALLAS were older, had a higher prevalence of congestive heart failure, and were more frequently on digoxin. The higher number of cardiovascular risk factors in patients of the PALLAS study and particularly the greater prevalence of congestive heart failure may be important factors contributing to the negative outcome of that study. Here, we provide evidence that dronedarone at clinically relevant concentrations inhibits *arterial* thrombus formation in a mouse model. The reduced number of first hospitalisations due to acute coronary syndromes in the ATHENA trial could well be explained by the pleiotropic anti-thrombotic effect described herein. Even though general survival was worse in dronedarone-treated patients and more cardiovascular events occurred in the PALLAS study, the number of myocardial infarctions did not differ. It may be possible that inhibition of arterial thrombus formation by dronedarone was equally active in the patients from PALLAS, but this beneficial effect was counterbalanced by the increase in other adverse events. To what extent dronedarone influences thrombus formation in a *low-shear* stress environment (e.g. venous system, left and right atrium) cannot be answered by this analysis since the pathophysiological mechanisms between arterial and venous thrombus formation are markedly different.

In summary, the present study demonstrates that dronedarone, at plasma concentrations similar to those occurring in humans, delays arterial thrombosis in vivo most probably through inhibition of platelet aggregation and enhancement of fibrinolysis. These findings thus offer novel insights into the possible reduction in ACS observed with dronedarone.

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Conflict of interest The current study was supported by Sanofi-Aventis with a restricted research grant. Dr Steffel and Dr Duru have received consulting honoraria from Sanofi-Aventis.

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